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(54) Inhibitors of cellular nicotinamide mononucleotide formation and their use in cancer therapy

(57) New biologically active substances are described which inhibit the cellular formation of niacinamide mononucleotide, an essential intermediate of the NAD(P) biosynthesis in the cell. These substances can represent the active ingredient of a pharmaceutical composition for the treatment of cancers, leukaemias or for immunosuppression. Furthermore, screening methods are described as a tool for detecting the above active substances, and for examination of a given cell type for its dependency on niacinamide as a precursor for NAD synthesis.

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Description

Field of the Invention

[0001] The present invention relates to new biologically active substances which inhibit the cellular formation of niacinamide mononucleotide, which is one of the essential intermediates in the NAD(P) biosynthesis in the cell. The invention further concerns pharmaceutical compositions containing these substances and their use, especially in the treatment of cancers, leukaemias or for immunosuppression. The invention also provides screening methods as a tool for detecting the above active substances, and for examination of cell types with respect to their NAD(P) synthesis pathway.

Technical Background of the Invention

[0002] NAD is synthesized in mammalian cells by three different pathways starting either from tryptophan via quinolinic acid, from niacin (also referred to as nicotinic acid) or from niacinamide (also referred to as nicotinamide), as shown in **Figure 1**.

[0003] The addition of a phosphoribosyl moiety results in the formation of the corresponding mononucleotides, niacin mononucleotide (dNAM) and niacinamide mononucleotide (NAM). Quinolinic acid is utilised in a reaction with phosphoribosyl pyrophosphate (PRPP) to form niacin mononucleotide (dNAM). The enzyme catalysing this reaction, quinolinic acid phosphoribosyl transferase (❸), is found in liver, kidney and brain.

[0004] Niacin reacts with PRPP to form niacin mononucleotide (dNAM). The enzyme catalysing this reaction is niacin phosphoribosyl transferase (❷) and is widely distributed in various tissues. Both pathways starting either from tryptophan or from niacin as NAD precursors merge at the stage of the niacin mononucleotide formation.

[0005] Niacinamide reacts with PRPP to give niacinamide mononucleotide (NAM). The enzyme that catalyses this reaction is niacinamide phosphoribosyl transferase (❶). This enzyme is specific for niacinamide and is entirely distinct from niacin phosphoribosyl transferase (❷). It is also widely distributed in various tissues.

[0006] The subsequent addition of adenosine monophosphate to the mononucleotides results in the formation of the corresponding dinucleotides: Niacin mononucleotide and niacinamide mononucleotide react with ATP to yield niacin adenine dinucleotide (dNAD) and niacinamide adenine dinucleotide (NAD), respectively. Both reactions, albeit taking place on two different pathways, are catalysed by the same enzyme, NAD pyrophosphorylase (❹).

[0007] A further amidation step is needed to convert niacin adenine dinucleotide (dNAD) to niacinamide adenine dinucleotide (NAD). The enzyme which catalyses this reaction is NAD synthetase (❺). NAD is the immediate precursor of niacinamide adenine dinucleotide phosphate (NADP). The reaction is catalysed by NAD kinase (❻). For details see, for example, Cory, J.G. Purine and pyrimidine nucleotide metabolism. In: Textbook of Biochemistry and Clinical Correlations, 3rd edition, ed. Devlin, T., Wiley Brisbane 1992, pp529-574.

[0008] Normal cells can typically utilize both precursors niacin and niacinamide for NAD(P) synthesis, and in many cases additionally tryptophan or its metabolites, which has been demonstrated for various normal tissues: Accordingly, Murine glial cells (cortex and hippocampus = brain) use: niacin, niacinamide, and quinolinic acid (Grant et al. (1998), J. Neurochem. 70: 1759-1763). Human lymphocytes use niacin and niacinamide (Carson et al. (1987), J. Immunol. 138: 1904-1907; Berger et al. (1982), Exp. Cell Res. 137: 79-88). Rat liver cells use niacin, niacinamide and tryptophan (Yamada et al. (1983), Internat. J. Vit. Nutr. Res. 53: 184-191; Shin et al. (1995), Internat. J. Vit. Nutr. Res. 65: 143-146; Dietrich (1971), Methods Enzymol. 18B: 144-149). Human erythrocytes use niacin and niacinamide (Rocchigiani et al. (1991), Purine and pyrimidine metabolism in man VII, Part B, ed. Harkness et al., Plenum Press, New York, pp 337-340. Leukocytes of guinea pigs use niacin (Flechner et al. (1970), Life Science. 9: 153-162).

[0009] NAD(P) is involved in a variety of biochemical reactions which are vital to the cell and have therefore been thoroughly investigated. This key function of NAD(P) has evoked also some investigations in the past on the role of this compound for the development and growth of tumors, and as to what the NAD(P) metabolism could also be utilized to combat tumors. Indeed, substances aiming at the treatment of tumor diseases have been described which involve - concomitantly to other effects - also the decrease of NAD(P) levels in the cell. However, these substances primarily act by initiating the cellular synthesis of dinucleotide derivatives which structurally deviate from natural NAD. The biochemical consequences of this approach and the putative mechanisms of the resulting cell-damage are, therefore, manifold as outlined in the **Table 1**.

Table 1

Compounds	Mode of action	Ref.
6-aminonicotinamide	**Primary mechanism of action:**	
	Synthesis of 6-amino-NAD(P), a competitive inhibitor of NAD(P)-requiring enzymes, especially of 6-phosphogluconate dehydrogenase, an enzyme of the pentose-phosphate-pathway which provides the precursor of the nucleotide biosynthesis ribose-5-phosphate. **Resulting biochemical effects in the cell:** Inhibition of purine nucleotide de novo synthesis from [^{14}C]glycine. Decrease of intracellular purine (ATP, GTP) and pyrimidine (UTP, CTP) nucleotide pools resulting in the inhibition of DNA and RNA synthesis. Inhibition of PARP (an enzyme involved in the DNA repair). Reduction of the ATP to ADP ratio. Depression of intracellular NAD concentration.	1, 2, 3
tiazofurin, selenazofurin	**Primary mechanism of action:** Synthesis of the NAD analogs TAD, SAD which are potent inhibitors of inosine monophosphate dehydrogenase, an enzyme involved in the synthesis of purine nucleotides. **Resulting biochemical effects in the cell:** Depletion of GMP and accumulation of IMP resulting in an inhibition of DNA and RNA synthesis. Stimulation of NAD synthesis after short exposure (<24 h). Inhibition of NAD synthesis after prolonged exposure (>24 h), probably due to negative feedback inhibition of NAD synthesis by TAD/SAD which accumulate in the cell.	2, 4, 5
azaserine, 6-diazo-5-oxo-L-norleucine	**Primary mechanism of action:** Analogs of glutamine which block the enzymatic transfer of the amido group of glutamine. **Resulting biochemical effects in the cell:** Inhibition of IMP synthesis resulting in an inhibition of DNA and RNA synthesis. Inhibition of NAD synthesis from the precursor niacin at the following step: dNAD $\rightarrow$ NAD Mutagen, cancerogen.	6, 7
DNA-interacting compounds	**Primary mechanism of action:** Induction of DNA strand breaks.	4, 8, 9, 10

Table 1 (continued)

Compounds	Mode of action	Ref.
(e.g. N-methyl-N'-nitro-N-nitroso-guanidine)	**Resulting biochemical effects in the cell:** Multiple consequences of DNA damage. Activation of the DNA repair enzyme PARP resulting in a decline of the intracellular NAD content, since the substrate of PARP is NAD. Mutagen, cancerogen.	
Abbreviations: PARP, poly(ADP-ribose) polymerase; **NAD**, niacinamide adenine dinucleotide; **NADP**, niacinamide adenine dinucleotide phosphate; **dNAD**, niacin adenine dinucleotide; **ATP**, adenosine triphosphate; **ADP**, adenosine diphosphate; **GTP**, guanosine triphosphate; **GMP**, guanosine monophosphate; **UTP**, uridine triphosphate; **CTP**, cytosine trisphosphate; **DNA**, desoxyribonucleic acid; **RNA**, ribonucleic acid; **TAD**, tiazofurin adenine dinucleotide; **SAD**, selenazofurin adenine dinucleotide; **IMP**, inosine monophosphate.		

[0010] It is therefore not possible to make any predictions from these data on the biological effects of a primary and specific inhibition of the NAD biosynthesis in various cell types. In particular, it remains completely speculative whether this mechanism may be advantageous over the above utilisation of dinucleotide derivatives with regard to tumor selectivity of the cell damaging effect, the most important feature of a potential drug for tumor therapy.

[0011] JP-459555, published in 1970, describes the extraction of a structurally unknown constituent from potatoes, baker's yeast and bovine blood which inhibits respiration of tumor cells and NAD synthesis of erythrocytes. The inventors propose the use of this constituent for tumor therapy. However, the data presented in JP-459555 are far from making it clear or even probable that inhibition of NAD synthesis is useful for the therapy of cancer. The inventors rather come to the conclusion that the biological activity of the substance is multifaceted and not limited merely to the phenomenon of inhibition of NAD biosynthesis. In a study published later by the same research group (A. Kizu: Kyoto Furitsu Ika Daigaku Zasshi 80, pp. 14-24, 1971) it was shown that the extracted compounds (derivatives of glucose) inhibit respiration and glycolysis in tumor cells already within a few minutes in addition to inhibition of NAD synthesis. In fact, tumor cells treated with the extract for only 20 min suffered from such heavy damage that they did not grow in the abdominal cavity of mice in contrast to untreated control cells. In contrast to this finding, the present inventors have observed that compounds which promptly and selectively inhibit NAD synthesis in the cell exert a deleterious effect on tumor cells only after an exposure for 3-4 days, whereas an exposure for 20 min is completely ineffective irrespective of the concentration employed. Thus, it is very unlikely that it is NAD biosynthesis inhibition by which the extract disclosed in JP-459555 damages tumor cells. It is rather assumed that other mechanisms are primarily responsible for the cell death, while the reduction of the NAD levels is a secondary effect due to the general damage to the cell. The prompt deleterious effect on tumor cells as produced by this extract is, therefore, obviously due to a inhibition of cell respiration.

[0012] Also, the tumor preference of the cell killing effect of the extract, as described in JP-459555, can easily be explained by a characteristic feature of the respiration inhibiting effect of the extract, as this effect is marked in tumor cells but absent in liver cells. (A. Kizu, Figure 2). Thus, clearly JP-459555 did not disclose any means to affect tumor cells by NAD synthesis inhibition.

[0013] It was also known that DNA damaging cytotoxic compounds initiate a decrease of the cellular concentration of NAD. Some authors assumed that lowering of cellular NAD levels, with a resulting shortage of ATP within the cell, may play a role in the mechanism of cell death produced by these compounds (Daniel S. Martin and Gary K. Schwartz, Oncology Research, Vol. 9, pp. 1-5, 1997). The effect of these compounds on the NAD concentration within the cell results, however, indirectly from an enhanced NAD consumption by enzymes involved in DNA repair (see **Table 1**).

[0014] The primary effect of these compounds, namely damage to the DNA, has many consequences in addition to lowering cellular NAD levels. As known, the DNA is in control of the synthesis of many cellular constituents, like proteins and enzymes, which are of vital importance to the cell. Thus, the consequences of DNA damage are also manifold, lowering of the cellular NAD concentration being only one of them. The efficacy profile of a specific inhibition of the NAD biosynthesis can, therefore, not be concluded from observations made with these compounds.

[0015] Just as little information on what can be expected from a specific inhibition of the biosynthesis of NAD gives the symptomatology of niacinamide and niacin deficiency. These vitamins of the B group are precursors of the NAD biosynthesis as outlined above. Long term deficiency of these precursors results in a disease known as pellagra. Main symptoms are alterations of the skin and dementia. This syndrome shows no similarity to the chronic intoxication with any of the compounds discussed above.

[0016] Thus, in summary, the state of the art does not allow to draw conclusions as to what can be expected from a

primary and specific inhibition of the cellular synthesis of NAD because the compounds known to lower the cellular NAD concentration exert other primary effects which may affect cell survival by themselves. There exists no other reliable means to solve this question than the use of a specific inhibitor of NAD synthesis. But no such compound was available in the past.

[0017] Morton (R. K. Morton: Nature 181, pp. 540-543, 1958) proposes for human cancer therapy to aim at compounds which inhibit the NAD pyrophosphorylase (Enzyme❹ in **Figure 1**) as the activity of this enzyme was assumed to be the limiting factor of NAD synthesis. Note that the biosynthesis pathway from both niacin and niacinamide, and also from tryptophan would be blocked by an inhibition of the NAD pyrophosphorylase since it acts on a late step of the biosynthesis pathway where the initially separated pathways starting from the different precursors tryptophan, niacin or niacinamide have already been united or are equally affected. No specific inhibitor of this enzyme has been found until now. Thus, no evidence for the correctness of this assumption is available.

Detailed Description of the Invention

[0018] The present invention is based on the surprising finding that specific cell types can essentially utilize only niacinamide as a precursor for the cellular NAD(P) biosynthesis. Niacin or tryptophan which constitute alternative precursors in all other cell types investigated so far cannot, or at least not to a significant extent, be utilized. Accordingly, the present invention provides for biologically active substances which inhibit the cellular formation of niacinamide mononucleotide. Substances having this activity can easily be identified by the screening assay described below. Preferably, the present substances exhibit an inhibitory activity on cellular NAD biosynthesis from the precursor niacinamide at concentrations of $\leq 10\ \mu M$ of 50 %, more preferably 80 % and most preferably 90 % in such an assay.

[0019] In this connection it is noted that niacinamide, which has been taken up, for example, with the diet, has first to be channeled into the cell across the cell membran before it can be converted by niacinamide phosphoribosyl transferase into niacinamide mononucleotide. The present invention covers therefore not only inhibitors of the niacinamide phosphoribosyl transferase, but also substances that hinder or block the transport of niacinamide across the cell membrane.

[0020] With the compounds of the present invention it is possible for the first time to damage exclusively those cells which mainly use niacinamide as a precursor for NAD biosynthesis saving those cells which are additionally able to synthesize NAD from niacin or tryptophan (**Figure 1**). It turned out that by using these compounds many malignant cells are affected while non-malignant cells can be saved. The same applies to certain lymphocytes which play a role in immune reactions. This behaviour has not been observed before and is also completely surprising: Neither there was any indication in the prior art nor could it be expected on the basis of the known data that normal somatic cell which can typically use all three kinds of precursors loose their ability to accommodate tryptophan and niacin and become dependent only on niacinamide when turning malignant.

[0021] Thus, biologically active compounds which selectively block the niacinamide branch of the NAD biosnthesis, i.e. inhibit the formation of niacinamide mononucleotide on the cellular level, would offer a new approach for selective tumor therapy: Malignant cells dependent on niacinamide as a main or sole precursor would suffer from such damage and finally be destroyed due to the inhibition of niacinamide mononucleotide formation and the subsequent NAD(P) depletion. On the other hand, normal somatic cells can compensate for the inhibited niacinamide branch by still utilizing niacin and/or tryptophan as precursors thereby providing sufficient NAD levels to guarantee survival of the cells.

[0022] The compounds of this invention are the first which primarily and specifically inhibit the biosynthesis of NAD from niacinamide. Therefore, these compounds can be used as a tool for investigation on the effect of this primary event on the survival of tumor cells and other cells of the body.

[0023] Moreover, it was completely surprising that the new specific inhibitors of NAD synthesis via niacinamide which deplete NAD in tumor cells within hours did not quickly kill the cells as shown with the known NAD synthesis "inhibitors" (see **Table 1**) but rather produced a characteristic "delayed cell death" phenomenon in these cells: continued growth for up to 3 days was observed in presence of the new compounds before practically all cells underwent apoptotic cell death. It was additionally surprising that many non-malignant cells are very resistant to the apoptosis-inducing effect of the new specific inhibitors of NAD biosynthesis. For instance a 10000-fold higher concentration is necessary to kill human bone marrow cells compared to most tested human cancer cell lines. Thus, the "delayed cell death" characteristic can be used in an assay to screen for substances according to the present invention.

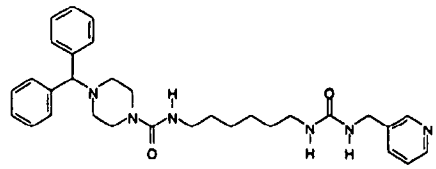
[0024] The ability of the compounds of the invention to inhibit the NAD biosynthesis from niacinamide can be shown with an easily reproducible test system which measures the incorporation of radioactive niacinamide into NAD and NADP. This assay provides a further sreening system to examine any chemical substance for its ability to selectively inhibit the cellular niacinamide mononucleotide formation. The assay allows to screen for and select the inhibitory compounds of the present invention without being bound to a particular structural characterization. Accordingly, there is no limitation on the chemical structure of these compounds as long as they exhibit said specific inhibitory activity, and any known preparation methods can be used.

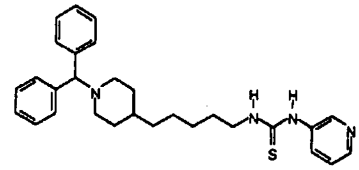
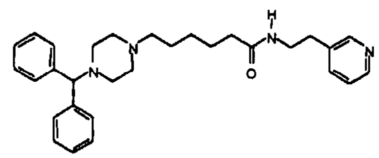
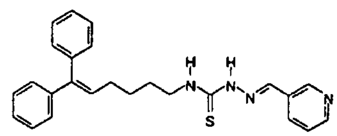
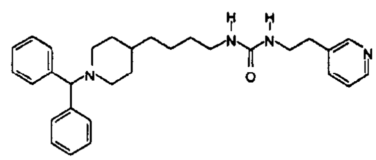
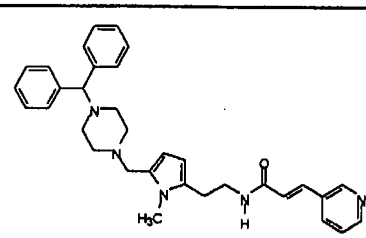
[0025] The fact that the death of tumor cells initiated by these compounds is indeed due solely to the inhibition of the NAD biosynthesis from niacinamide and not due to any other effect could be unequivocally verified: Addition of excess niacinamide to the extracellular medium in which the cells are grown *in vitro* completely reverses the apoptosis inducing effect of the new compounds.

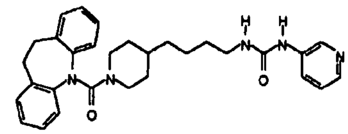
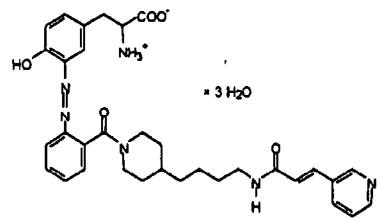
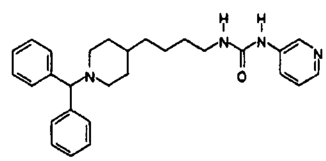
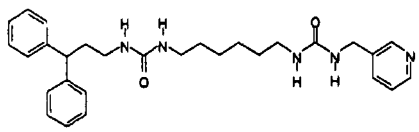
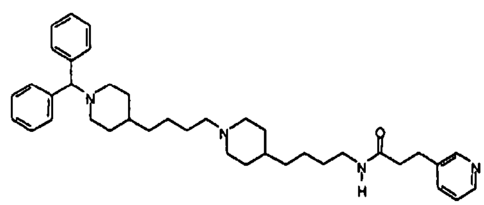
[0026] The effects of the compounds according to the invention on cell growth under high-density conditions have been investigated in order to closely simulate the *in vivo* situation of solid tumors. For this purpose, the inventors seeded high cell numbers and carried out high density cell culture experiments with the compounds shown in **Table 2** below. Cell growth was monitored at various times up to 10 days as described in the experimental part below. Human hepatocarcinoma cells (HepG2) were used, for example.

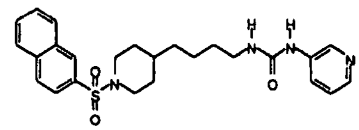
[0027] The time curve of the action of the compounds is characterised by the induction of "delayed cell death" which is clearly distinguished from a rapid decline of cell numbers occurring after the application of toxic compounds. The "delayed cell death" phenomenon is described using for example the results obtained with K22.339. **Figure 2** demonstrates the characteristic time curve of growth inhibition by substance K22.339. During incubation with K22.339 the number of HepG2 cells increased up to three days, after which the culture was no longer able to grow and cell numbers declined from day 7 to 10. Cell death occurred on day 4, and the cells gradually detached until day 10. In contrast, toxic compounds are less active in high density cultures and effective concentrations induced a rapid decrease in cell numbers observed as soon as one to three days of incubation. Using K22.339, however, a concentration of 0.3 μM was sufficient to bring about the full-blown effect. A still ten times higher concentration did neither show acute cytotoxicity, nor was it able to accelerate the time until the cell number gradually decreased. This characteristic action was referred to as delayed cell death.

Table 2

K-No.	Structure	DCD [μM]
K22.132	 4-benzhydryl-piperazine-1-carboxylic acid-[6-(3-pyridine-3-yl-methylureido)-hexyl]-amide	0.1

K-No.	Structure	DCD [µM]
K22.234	 1-[5-(1-benzhydryl-piperidine-4-yl)-pentyl]-3-pyridine-3-yl-thiourea	0.3
K22.265	 6-(4-benzhydryl-piperazine-1-yl)-hexanoic acid-(2-pyridine-3-yl-ethyl)-amide	3
K22.299	 1-(6,6-diphenyl-5-hexenyl)-3-(pyridine-3-yl-methylene-amino)-thiourea	0.3
K22.339	 1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-3-(2-pyridine-3-yl-ethyl)-urea	0.1
K22.350	 N-{2-[5-(4-benzhydryl-piperazine-1-yl-methyl)-1-methyl-1H-pyrrole-2-yl]-ethyl}-3-pyridine-3-yl-acrylamide	1

K-No.	Structure	DCD [µM]
K22.387	 1-{4-[1-(10,11-dihydro-dibenzene[b,f]azepine-5-carbonyl)-piperidine-4-yl]-butyl}-3-pyridine-3-yl-urea	0.01
K22.408	 2-amino-3-[4-hydroxy-3-(2-{4-[4-(3-pyridine-3-yl-acryloyl-amino)-butyl]-piperidine-1-carbonyl}-phenylazo)-phenyl]-propanoic acid trihydrate	1
K22.130	 1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-3-pyridine-3-yl-urea	1
K22.158	 1-(3,3-diphenylpropyl)-3-[6-(3-pyridine-3-yl-methylureido)-hexyl]-urea	1
K22.316	 N-(4-{1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-piperidine-4-yl}-butyl)-3-pyridine-3-yl-propanoic acid amide	1

K-No.	Structure	DCD [μM]
K22.365	 1-{4-[1-(naphthalin-2-sulfonyl)-piperidine-4-yl)-butyl}-3-pyridine-3-yl-urea	3

[0028] The DCD-value was defined as the minimum concentration of the respective compound, which - despite initial growth of the culture - induced cell death below the number of initially seeded cells. All compounds were active on high density cultures of HepG2 cells at concentrations of 3 μM or lower. It is therefore preferred that the compounds of the present invention are active in the "delayed cell death" test at a concentration of 3 μM or lower, particularly preferred at a concentration of 1 μM or lower. The compounds listed in **Table 2** were prepared according to standard methods known in the art.

[0029] The time course of the effect of the compounds suggested that there was no acute unspecific toxicity on the tumor cells. This time course is in sharp contrast to the results described in JP-459555. The authors wrote that a 20 min incubation period of tumor cell with the disclosed extract was sufficient to induce cell death. On the other hand, the present compounds seem to impose some physiological limitation on the cells which should leave enough time for them to sense the restriction and commit suicide before uncontrolled necrosis could take place. "Delayed cell death" induced by the compounds occurred in the form of apoptosis which is a favourable way of removing no longer viable cells, because it avoids the unregulated release of cell contents to the surrounding tissue. The "delayed cell death" induced by the compounds can be antagonised by the addition of niacinamide.

[0030] The effect of the compounds on the synthesis of NAD(P) starting from niacinamide was investigated using for example the tumor cell line HepG2 according to the technique described in the experimental part below. As shown in **Table 3**, the compounds completely inhibited the *de novo* synthesis of NAD(P) from its precursor niacinamide.

Table 3

K-No.	NAD(P) pmol/10^6 cells	NAD(P) pmol/mg protein	% of Control
Control	302.1	186.4	100
K22.132	4.1	2.5	1.4
K22.234	3.4	2.1	1.1
K22.265	7.3	4.5	2.4
K22.299	1.6	1.0	0.5
K22.339	2.5	1.6	0.8
K22.350	2.7	1.7	0.9
K22.387	2.6	1.6	0.9
K22.408	4.9	3.0	1.6

[0031] The inhibition of the NAD(P) synthesis from niacinamide by these compounds was investigated in HepG2 cells as described in Material and Methods. The compounds were used at a concentration of 10^{-5} M. As a control, vehicle treated or untreated cells were used.

[0032] In these experiments, a pre-incubation period of 17 hrs with the compounds was used, but it turned out that the pre-incubations period could be shortened, for example to 2 hrs, or skipped completely without affecting the inhibtion profile. Further analysis of the radiolabeled niacinamide metabolites revealed that the compounds exclusively blocked the formation of the niacinamide mononucleotide. **Figure 3** shows representative chromatograms of [^{14}C]niacinamide metabolites extracted from vehicle - and K22.234-treated HepG2 cells. Similar results werde obtained with the other compounds.

[0033] By comparing the two chromatograms in **Figure 3**, it is clearly evident that the compounds inhibited the synthesis of NAD(P) from its precursor niacinamide. The radiolabelled niacin seen in the extract of the vehicle- and the compound-treated cells resulted from the enzymatic deamidation of [^{14}C]niacinamide. The peaks of NAD and niacin on the chromatograms are close together, but the inventors verified the identification by the second thin-layer chromatography system, as described in Materials and Methods. Since an accumulation of the intermediate niacinamide mononucleotide was not detected in the compound-treated cells, the compounds inhibit the NAD(P) biosynthesis at the step of the formation of niacinamide mononucleotide. Thus, the compounds inhibit the enzyme niacinamide pyrophosphate phosphoribosyl transferase [EC 2.4.2.12]; a second name of this enzyme is niacinamide mononucleotide pyrophosphorylase) and/or the niacinamide transport into the cell. Performing the same kind of experiments using [^{14}C]niacin instead of [^{14}C]niacinamide as precursor revealed that the compounds do not block the NAD(P) synthesis from the precursor niacin in cells which are able to use the niacin pathway. The observation that the niacin pathway of NAD(P) synthesis is not blocked by the compounds makes it very unlikely that pathway starting from tryptophan is suppressed by the compounds.

[0034] Before the advent of the present invention, no compounds have been described which exclusively inhibit the NAD(P) synthesis at this step. Accordingly, this mode of action is entirely different from that proposed by Morton (Nature 181:540-543, 1958) for tumor therapy. He suggested to inhibit the enzyme NAD pyrophosphorylase, but the inhibition of this enzyme would block the synthesis of NAD(P) from all three precursors (niacinamide, niacin and tryptophan, compare **Figure 1**). It was absolutely surprising that the inhibition of NAD synthesis at the step of the formation of niacinamide mononucleotide in the niacinamide pathway was sufficient to kill most tumor cells.

[0035] The present compounds are the first tools to investigate the importance of the niacinamide pathway in tumor cells. The present inventors found that most tumour cells, for example, HepG2 (liver carcinoma), U-87 MG (glioblastoma, astrocytoma), U-373 MG (glioblastoma, astrocytoma), Caki-1 (renal clear cell carcinoma), KG-la (myelogenic leukaemia), HL-60 (promyelocytic leukaemia), A549 (lung carcinoma), MCF-7 M1 (breast carcinoma), PC3 (prostate carcinoma), can utilize only on the niacinamide pathway of NAD(P) synthesis. From this finding it is concluded that these compounds can be used for the therapy of the corresponding cancers (e.g. breast, prostate, lung, colon, cervix, ovary, skin, CNS, bladder, pancreas and leukemia and lymphoma). The above cell lines are known and commercially available.

[0036] Furthermore, blocking only the niacinamide pathway protect those cells from the effect of the compounds which can also use niacin or tryptophan as precursor. These cells are typical healthy somatic cells, e.g. liver cells, Kupffer cells, lung epithelial cells, renal epithelial cells, lymphocytes, colon epithelial cells or dermal fibroblasts. In regard to side effects, this essentially sole inhibition of the niacinamide pathway is of enormous advantage for the treatment of cancer.

[0037] For the detection of specific inhibitors of niacinamide mononucleotide formation the following screening assay is especially useful which comprises the following steps: incubating cultured cells selected from HepG2 cells, U-87 MG cells, MCF-7 M1 cells, Caki-1 cells, HL-60 cells, PC3 cells, U-373 MG cells, A549 cells and KG-1a cells in the presence of [^{14}C]niacinamide and a substance to be tested for its activity to inhibit the cellular formation of niacinamide mononucleotide; effecting lysis of the cells; isolating and separating the [^{14}C]-labelled compounds and measuring the amount of formed labelled niacinamide mononucleotide, NAD and NADP. More concretely, cultured cells are seeded in a defined density in culture dishes, followed by incubation in the presence of a test compound and by the addition of [^{14}C]niacinamide for about 0.1 to 6 hrs. The cells are then lysed with perchloric acid and the resulting extract is neutralised. Finally, the [^{14}C]-labeled compounds are separated by thin-layer chromatography on cellulose matrices followed by UV detection and autoradiography. Non-radioactive niacinamide derivatives are used as standards. This assay allows simple and effective screening and selection of the compounds according to the invention.

[0038] The present invention also provides a method for assaying the dependency of a given cell type on niacinamide as a precursor for NAD synthesis. This method allows to determine which (malignant) cell types are particularly sensitive to the substances according to the invention and can be helpful to develop a suitable regimen for combatting various tumors. Accordingly, such an assay would comprise incubating cells to be tested in the presence of a substance according to the invention in a medium containing only niacinamide as a NAD synthesis precursor; and perfoming a cytotoxicity assay after the incubation period. Such a cytotoxicity assay could, for example, be the "high desitiy cell test" described in the experimental part.

Example

Material and Methods

Reagents:

[0039]

Trypsin/EDTA:	0.05 % (w/v) trypsin (Difco, Detroit, USA) + 0.016 % (w/v) EDTA (Sigma, Deisenhofen, Germany).
[^{14}C]Niacinamide:	ARC794, American Radiolabeled Chemicals Inc., St. Louis, MO, USA, 0.25 mCi/ml; specific activity 50 mCi/mmol.
Lysis buffer:	0.5 M perchloric acid (Merck, Darmstadt, Germany).
Neutralisation reagent:	0.5 mM potassium chloride, 2.0 M potassium hydroxide, dissolved in purified water. The chemicals were obtained from Merck, Darmstadt, Germany.
TLC foils:	Cellulose F, Art. 1.05565, Merck, Darmstadt, Germany Poly(ethyleneimine) Cellulose F, Art. 1.05579, Merck Darmstadt, Germany.
TLC solvents:	0.05 M lithium chloride or 3 parts 1 M ammonium acetate pH 5.0 + 7 parts ethanol (Merck, Darmstadt, Germany).
Standards:	10 to 20 mg/ml solutions of the following niacinamide derivatives were prepared: niacin, niacinamide, niacin mononucleotide (dNAM), niacinamide mononucleotide (NAM), niacin adenine dinucleotide (dNAD), niacinamide adenine dinucleotide (NAD), niacinamide adenine dinucleotide phosphate (NADP). All standards were purchased from Sigma, Deisenhofen, Germany.
TCA solution:	500 g trichloro-acetic acid, dissolved in H2O ad 2 l (25% w/v).
10 mM Tris buffer:	121.1 mg trishydroxymethyl aminomethane (Sigma, Deisenhofen, Germany) dissolved in 100 ml H2O, titrated to pH = 10.4 with NaOH.
SRB solution:	400 mg sulforhodamine B (Sigma, Deisenhofen, Germany), dissolved in 100 ml of 1% (v/v) acetic acid.
Test substances:	K22-compounds were synthesised by the department of chemistry at Klinge Pharma GmbH, Munich, Germany. Stock solutions: a 10 mM solution was prepared in dimethylsulfoxide (DMSO) and stored at -18°C; further dilution steps were done in ethanol.
Cell line:	The human-derived tumour cell line HepG2 (liver carcinoma) was obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, USA.

Growth medium:

[0040] Richter's Improved Minimal Essential Medium, Zinc Option (IMEM-ZO), was purchased from Gibco BRL, Life Technologies (Eggenstein, Germany) (Richter, A., Sanford, K.K. and Evans, V.J. (1972) , J. Natl. Cancer Inst. 49: 1705-1712). The medium powder was dissolved in deionised water, the pH titrated to 7.2 with HCl / NaOH and sterilised by filtration. The medium was supplemented with 5 % or 10 % fetal calf serum (FCS), PAN Systems GmbH, Aidenbach, Germany; 100 µg/l insulin (Boehringer, Mannheim, Germany) and 50,000 IU/l penicillin + 50 mg/l streptomycin (Sigma, Deisenhofen, Germany).

[0041] HEPES-buffered IMEM-ZO: This medium was used for incubation of HepG2 cells with the radiolabeled precursor. In contrast to the above-described Richter's IMEM-ZO, it did not contain niacinamide, $NaHCO_3$ and FCS. This medium was specifically prepared by Gibco BRL, Life Technologies (Eggenstein, Germany). The medium was buffered with 20 mM HEPES (Sigma, Deisenhofen, Germany) and the pH was adjusted to 7.2. The medium was sterilized by filtration.

Determination of NAD(P) synthesis from [^{14}C]niacinamide:

Cell culture:

[0042] The cells were detached from 75 cm^2 flasks by removing the growth medium and adding 3 ml trypsin/EDTA solution to each flask. After about 5 minutes incubation at 37°C, when the cells were detached from the surface of the dishes, trypsinization was stopped by adding 3 ml Richter's IMEM-ZO medium containing 10 % FCS. The cells were suspended by repeated pipetting. For predilution, an aliquot of 20 µl was added to 10 ml Casyton isotonic solution (No. 043-90037P, Schärfe System, Reutlingen, Germany) using a Sysmex Auto Dilutor Type AD-260 (Toa, Medical Elec-

tronics Co. Ltd., Kobe, Japan). The cell number was determined by electronic cell volume measurements and counting with a CASY 1 Cell Counter + Analyzer System, Model TTC (Schärfe System, Reutlingen, Germany) equipped with a 60 µm capillary. Following dilution in IMEM-ZO containing 10 % FCS, the cells were finally seeded at a density of $4x10^6$/ 10 ml per sample in Ø 10 cm tissue culture dishes (Greiner, Frickenhausen, Germany) and incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air.

[0043] After one day, when the cells were adherent to the dishes, the cultures were replenished with IMEM-ZO containing 5 % FCS plus the test compound or the vehicle. Concentrations of organic solvents in the medium after addition of the test substance did not exceed 0.1 % in any case. The cells were then incubated at 37°C for 17 hours in a humidified atmosphere of 5 % CO_2 in air. This preincubation period is not necessary to achieve a distinct inhibitory action of the compounds and can be shortened for example to 2 or 0 hours. After this period of time, the medium was again discarded and 4 ml HEPES-buffered IMEM-ZO containing the test compound or the vehicle and 0.5 µCi/ml [^{14}C] niacinamide were added to each culture for an additional 5 hours at 37°C and 100 % humidity. Just before the cells were harvested with a cell scraper and transferred to 15 ml polypropylene tubes, a 100 µl aliquot was taken from the incubation medium to determine the radioactivity. The culture dishes were rinsed with 4 ml saline supplemented with 10 mM niacinamide and the solutions were pooled with the respective cell suspension. The cells were collected by centrifugation at 250 g for 5 minutes at 4°C.

Extraction of pyridine nucleotides

[0044] Pyridine nucleotides were extracted by a modification of the procedure of Chatterjee et al. (Chatterjee, S., Hirschler, N.V., Petzold, S.J., Berger, S.J. and Berger, N.A. (1989) Mutant Cells Defective in Poly(ADP-ribose) Synthesis due to Stable Alterations in Enzyme Activity or Substrate Availability. Exp. Cell Res. 184: 1-15). Briefly, each cell pellet was suspended in 200 µl ice-cold 0.5 M perchloric acid and incubated on ice for 20 minutes. After this period of time, the acid extracts were neutralized by adding 55 µl of a KCl/KOH solution and centrifuged at 2500 g for 10 minutes at 4°C. Supernatants were collected and stored at-20°C until separation by chromatography. A 10 µl aliquot was taken from each supernatant to measure the total amount of radioactivity in the cell extract.

Thin-layer chromatography

[0045] The ^{14}C-labeled components of the cell extracts were separated and identified using two thin-layer chromatography (TLC) systems. 2 µl of each cell extract was transferred to a cellulose and a poly(ethyleneimine) (PEI) cellulose TLC foil using a DC-Probenautomat III (CAMAG, Muttenz, Switzerland). The cellulose foils were developed using 1 M NH4 acetate:ethanol (3:7) as solvent (Pinder, S., Clark, J.B. and Greenbaum, A.L. (1971) The Assay of Intermediates and Enzymes Involved in the Synthesis of the Nicotinamide Nucleotides in Mammalian Tissues. Methods in Enzymology. Academic Press, New York. Vol. XVIIIB pp. 20-46). The PEI cellulose plates were developed with 0.05 M lithium chloride (Barton, R.A., Schulman, A., Jacobson, E.L. and Jacobson, M.K. (1977) Chromatographic Separation of Pyridine and Adenine Nucleotides on Thin Layers of Poly(ethyleneimine) Cellulose. J. Chromatogr. 130: 145-150).

[0046] The chromatograms were run with added non-radioactive standards of NAD, NADP, NAM, dNAM, dNAD, niacin and niacinamide, and the spots were identified by UV absorption. See **Table 4** for R_F values. Results are expressed as mean ± S.D. For autoradiography, the chromatograms were exposed to an imaging plate BAS-IIIs (Fuji Photo Film Co., Ltd., Japan) in a hypercassette (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany) for at least two days. To avoid high background activity, the cassette was placed in a lead box. After exposure, the imaging plate was read in the bio-imaging analyzer FUJIFILM BAS-1500 (Fuji Photo Film Co., Ltd., Japan). The portion of each [^{14}C]-labeled component in the cell extracts was determined as percentage of total radioactivity with the software TINA 2.0 (raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Table 4

Standard	**Matrix / Solvent**	
	PEI cellulose / LiCl	Cellulose / NH_4 acetate:ethanol
Niacin	0.45 ± 0.03 (n = 12)	0.73 ± 0.04 (n = 14)
Niacinamide	0.77 ± 0.01 (n = 14)	0.80 ± 0.04 (n = 14)
dNAM	0.18 ± 0.04 (n = 4)	0.19 ± 0.04 (n = 4)
NAM	0.52 ± 0.08 (n = 3)	0.19 ± 0.05 (n = 3)
dNAD	0.07 ± 0.01 (n = 10)	0.09 ± 0.01 (n = 10)

Table 4 (continued)

Standard	Matrix / Solvent	
	PEI cellulose / LiCl	Cellulose / NH_4 acetate:ethanol
NAD	0.37 ± 0.02 (n = 14)	0.11 ± 0.02 (n = 14)
NADP	0.02 ± 0.01 (n = 13)	0.04 ± 0.01 (n = 13)

[0047] The amount of ^{14}C-labeled derivatives was calculated by multiplying the total radioactivity of the cell extract by the percentage recovered in each derivative. The results of the assay as shown in **Table 3** above are expressed as pmol [^{14}C]NAD(P) per 10^6 cells and as pmol [^{14}C]NAD(P) per mg protein. Cell count and cellular protein were determined from cultures prepared in parallel without radioactive precursors.

Protein determination:

[0048] The cellular protein was determined with the bicinchoninic acid (BCA) assay purchased from Pierce, Rockford, IL, USA, according to the manufacturer's instructions. The colour of the samples produced from the reaction was measured spectrophotometrically (COBAS FARA *II*, F. Hoffmann-La Roche AG, Basel, Switzerland).

Determination of cell growth under high-density conditions: Cell culture

[0049] The cells were detached from 75 cm2 flasks by removing the growth medium and adding 3 ml trypsin/EDTA solution to each well. After 5 minutes incubation at 37°C, when the cells were detached from the surface of the dishes, trypsinisation was stopped by adding 3 ml Richter's IMEM-ZO medium containing 10 % FCS. The cells were suspended by repeated pipetting. For predilution an aliquot of 20 µl was added to 10 ml Casyton isotonic solution (No. 043-90037P, Schärfe System, Reutlingen, Germany) using a Sysmex Auto Dilutor Type AD-260 (Toa, Medical Electronics Co. Ltd., Kobe, Japan). The cell number was determined by electronic cell volume measurements and counting with a CASY 1 Cell Counter + Analyser System, Model TTC (Schärfe System, Reutlingen, Germany) equipped with a 60 µm capillary. Following dilution in growth medium the cells were finally seeded at a density of 200,000 cells per ml and well in 24-well culture dishes (Greiner, Frickenhausen, Germany). Additionally, three negative control wells were incubated in growth medium without any cells.

[0050] After one day, when the cells were adherent to the dishes, the cultures were replenished with fresh medium containing 5 % FCS plus different concentrations of the test substances or the vehicle. Triplicate samples were prepared from each concentration and the cells were incubated at 37°C in a humidified atmosphere of 5 % CO_2 in air. Concentration of organic solvents in the medium after addition of test substances did not exceed 0.1 % in any cases.

Sulforhodamine B assay (SRB)

[0051] Determination of cell growth was performed by unspecific protein staining with sulforhodamine B according to Skehan et al. (Skehan, P. et al. (1990) New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. J. Natl. Cancer Inst. 82: 1107-1112).

[0052] The drug incubation period of the cells was stopped by the addition of 250 µl of ice cold TCA solution into the growth medium. After one hour incubation in the refrigerator, the supernatant was discarded, the dishes were rinsed five times with deionised water, dried at room temperature (RT) and finally stored in the refrigerator until staining. 0.5 ml of SRB solution was pipetted into each well and incubated at room temperature for 30 minutes; thereafter, the staining solution was decanted, the dishes were washed four times with 1% (v/v) acetic acid and dried again at RT. SRB stain unspecifically bound to protein was released by adding 1 ml of 10 mM Tris buffer per well and gentle shaking for 5 minutes. 100 µl aliquots of each well were transferred to a 96-well microtiter plate and the light extinction at 515 nm wavelength was read in an ELISA-reader (Bio-Tek, Deelux, Gödensdorf, Germany). The mean value of negative control wells was subtracted from the test sample readings.

THERAPEUTIC ADMINISTRATION FORMS

[0053] The production of medicaments with an amount of one or more compounds according to the invention and/or their use in the application according to the invention occurs in the customary manner by means of common pharmaceutical technology methods. For this, the active ingredients as such or in the form of their salts are processed together with suitable, pharmaceutically acceptable adjuvents and carriers to medicinal forms suitable for the various

indications and types of application. Thereby, the medicaments can be produced in such a manner that the respective desired release rate is obtained, for example a quick flooding and/or a sustained or depot effect.

[0054] Preparations for parenteral use, to which injections and infusions belong, are among the most important systemically employed medicaments for tumor treatment as well as for other indications.

[0055] Preferably, injections are administered for the treatment of tumors. These are prepared either in the form of vials or also as so-called ready-to-use injection preparations, for example as ready-to-use syringes or single use syringes in addition to perforation bottles for multiple withdrawals. Administration of the injection preparations can occur in the form of subcutaneous (s.c.), intramuscular (i.m.), intravenous (i.v.) or intracutaneous (i.c.) application. The respective suitable injection forms can especially be produced as solutions, crystal suspensions, nanoparticular or colloid-disperse systems, such as for example, hydrosols.

[0056] The injectable formulations can also be produced as concentrates which can be adjusted with aqueous isotonic dilution agents to the desired active ingredient dosage. Furthermore, they can also be produced as powders, such as for example lyophilisates, which are then preferably dissolved or dispersed immediately before application with suitable diluents. The infusions can also be formulated in the form of isotonic solutions, fat emulsions, liposome formulations, microemulsions and liquids based on mixed micells, for example, based on phospholipids. As with injection preparations, infusion formulations can also be prepared in the form of concentrates to dilute. The injectable formulations can also be applied in the form of continuous infusions as in stationary as well as in outpatient therapy, for example in the form of mini-pumps.

[0057] Albumin, plasma expanders, surface active compounds, organic solvents, pH influencing compounds, complex forming compounds or polymeric compounds can be added to the parenteral medicinal forms, especially as substances for influencing the adsorption of the active ingredients to protein or polymers or also with the aim of decreasing the adsorption of the active ingredient to materials such as injection instruments or packaging materials, for example plastic or glass.

[0058] The active ingredients can be bound to nanoparticles in the preparations for parenteral use, for example on finely dispersed particles based on poly(meth)acrylates, polyacetates, polyglycolates, polyamino acids or polyether urethanes. The parenteral formulations can also be constructively modified as depot preparations, for example on the multiple unit principle, where the active ingredients are incorporated in a most finely distributed and/or dispersed, suspended form or as crystal suspensions, or on the single unit principle, where the active ingredient is enclosed in a medicinal form, for example, a tablet or a seed which is subsequently implanted. Often, these implantations or depot medicaments in single unit and multiple unit medicinal forms consist of so-called biodegradable polymers, such as for example, polyether urethanes of lactic and glycolic acid, polyether urethanes, polyamino acids, poly(meth)acrylates or polysaccharides.

[0059] Sterilized water, pH value influencing substances, such as for example organic and inorganic acids or bases as well as their salts, buffer substances for setting the pH value, agents for isotonicity, such as for example sodium chloride, monosodium carbonate, glucose and fructose, tensides and/or surface active substances and emulsifiers, such as for example, partial fatty acid esters of polyoxyethylene sorbitan (Tween®) or for example fatty acid esters of polyoxethylene (Cremophor®), fatty oils such as for example peanut oil, soybean oil and castor oil, synthetic fatty acid esters, such as for example ethyl oleate, isopropyl myristate and neutral oil (Miglyol®) as well as polymer adjuvents such as for example gelatin, dextran, polyvinylpyrrolidone, organic solvent additives which increase solubility, such as for example propylene glycol, ethanol, N,N-dimethylacetamide, propylene glycol or complex forming compounds such as for example citrates and urea, preservatives, such as for example hydroxypropyl benzoate and hydroxymethyl benzoate, benzyl alcohol, anti-oxidants, such as for example sodium sulfite and stabilizers, such as for example EDTA, are suitable as adjuvents and carriers in the production of preparations for parenteral use.

[0060] In suspensions, addition of thickening agents to prevent the settling of the active ingredients from tensides and peptizers, to secure the ability of the sediment to be shaken, or complex formers, such as EDTA, ensues. This can also be achieved with the various polymeric agent complexes, for example with polyethylene glycols, polystyrol, carboxymethylcellulose, Pluronics® or polyethylene glycol sorbitan fatty acid esters. The active ingredient can also be incorporated in liquid formulations in the form of inclusion compounds, for example with cyclodextrins. As further adjuvents, dispersion agents are also suitable. For production of lyophilisates, builders are also used, such as for example mannite, dextran, saccharose, human albumin, lactose, PVP or gelatin varieties.

[0061] As long as the active ingredients are not incorporated in the liquid medicinal formulations in the form of a base, they are used in the form of their acid addition salts, hydrates or solvates in the preparations for parenteral use.

[0062] A further systemic application form of importance is peroral administration as tablets, hard or soft gelatin capsules, coated tablets, powders, pellets, microcapsules, oblong compressives, granules, chewable tablets, lozenges, gums or sachets. These solid peroral administration forms can also be prepared as sustained action and/or depot systems. Among these are medicaments with an amount of one or more micronized active ingredients, diffusions and erosion forms based on matrices, for example by using fats, wax-like and/or polymeric compounds, or so-called reservoir systems. As a retarding agent and/or agent for controlled release, film or matrix forming substances, such as

for example ethylcellulose, hydroxypropylmethylcellulose, poly(meth)acrylate derivatives (for example Eudragit®), hydroxypropylmethylcellulose phthalate are suitable in organic solutions as well as in the form of aqueous dispersions. In this connection, so-called bio-adhesive preparations are also to be named in which the increased retention time in the body is achieved by intensive contact with the mucus membranes of the body. An example of a bio-adhesive polymer is the group of Carbomers®.

[0063] For sublingual application, compressives, such as for example non-disintegrating tablets in oblong form of a suitable size with a slow release of active ingredient, are especially suitable. For purposes of a targeted release of active ingredients in the various sections of the gastrointestinal tract, mixtures of pellets which release at the various places are employable, for example mixtures of gastric fluid soluble and small intestine soluble and/or gastric fluid resistant and large intestine soluble pellets. The same goal of releasing at various sections of the gastrointestinal tract can also be conceived by suitably produced laminated tablets with a core, whereby the coating of the agent is quickly released in gastric fluid and the core of the agent is slowly released in the small intestine milieu. The goal of controlled release at various sections of the gastrointestinal tract can also be attained by multilayer tablets. The pellet mixtures with differentially released agent can be filled into hard gelatin capsules.

[0064] Anti-stick and lubricant and separating agents, dispersion agents such as flame dispersed silicone dioxide, disintegrants, such as various starch types, PVC, cellulose esters as granulating or retarding agents, such as for example wax-like and/or polymeric compounds on the basis of Eudragit®, cellulose or Cremophor® are used as a further adjuvents for the production of compressives, such as for example tablets or hard and soft gelatin capsules as well as coated tablets and granulates.

[0065] Anti-oxidants, sweetening agents, such as for example saccharose, xylite or mannite, masking flavors, aromatics, preservatives, colorants, buffer substances, direct tableting agents, such as for example microcrystalline cellulose, starch and starch hydrolysates (for example Celutab®), lactose, polyethylene glycols, polyvinylpyrrolidone and dicalcium phosphate, lubricants, fillers, such as lactose or starch, binding agents in the form of lactose, starch varieties, such as for example wheat or corn and/or rice starch, cellulose derivatives, for example methylcellulose, hydroxypropylcellulose or silica, talcum powder, stearates, such as for example magnesium stearate, aluminum stearate, calcium stearate, talc, siliconized talc, stearic acid, acetyl alcohol and hydrated fats are used.

[0066] In this connection, oral therapeutic systems constructed especially on osmotic principles, such as for example GIT (gastrointestinal therapeutic system) or OROS (oral osmotic system), are also to be mentioned.

[0067] Effervescent tablets or tabs. both of which represent immediately drinkable instant medicinal forms which are quickly dissolved or suspended in water are among the perorally administratable compressives. Among the perorally administratable forms are also solutions, for example drops, juices and suspensions, which can be produced according to the above given method, and can still contain preservatives for increasing stability and optionally aromatics for reasons of easier intake, and colorants for better differentiation as well as antioxidants and/or vitamins and sweeteners such as sugar or artificial sweetening agents. This is also true for inspisated juices which are formulated with water before ingestion. Ion exchange resins in combination with one or more active ingredients are also to be mentioned for the production of liquid ingestable forms.

[0068] A special release form consists in the preparation of so-called floating medicinal forms, for example based on tablets or pellets which develop gas after contact with body fluids and therefore float on the surface of the gastric fluid. Furthermore, so-called electronically controlled release systems can also be formulated by which active ingredient release can be selectively adjusted to individual needs.

[0069] A further group of systemic administration and also optionally topically effective medicinal forms are represented by rectally applicable medicaments. Among these are suppositories and enema formulations. The enema formulations can be prepared based on tablets with aqueous solvents for producing this administration form. Rectal capsules can also be made available based on gelatin or other carriers.

[0070] Hardened fat, such as for example Witepsol®, Massa Estarinum®, Novata®, coconut fat, glycerol-gelatin masses, glycerol-soap-gels and polyethylene glycols are suitable as suppository bases.

[0071] For long-term application with a systematic active ingredient release up to several weeks, pressed implants are suitable which are preferably formulated on the basis of so-called biodegradable polymers.

[0072] As a further important group of systemically active medicaments, transdermal systems are also to be emphasized which distinguish themselves, as with the above-mentioned rectal forms, by circumventing the liver circulation system and/or liver metabolism. These plasters can be especially prepared as transdermal systems which are capable of releasing the active ingredient in a controlled manner over longer or shorter time periods based on different layers and/or mixtures of suitable adjuvents and carriers. Aside from suitable adjuvents and carriers such as solvents and polymeric components, for example based on Eudragit®, membrane infiltration increasing substances and/or permeation promoters, such as for example oleic acid, Azone®, adipinic acid derivatives, ethanol, urea, propylglycol are suitable in the production of transdermal systems of this type for the purpose of improved and/or accelerated penetration.

[0073] As topically, locally or regionally administration medicaments, the following are suitable as special formula-

tions: vaginally or genitally applicable emulsions, creams, foam tablets, depot implants, ovular or transurethral administration installation solutions. For opthalmological application, highly sterile eye ointments, solutions and/or drops or creams and emulsions are suitable.

[0074] In the same manner, corresponding otological drops, ointments or creams can be designated for application to the ear. For both of the above-mentioned applications, the adminstration of semi-solid formulations, such as for example gels based on Carbopols® or other polymer compounds such as for example polyvinylpyrolidone and cellulose derivatives is also possible.

[0075] For customary application to the skin or also to the mucus membrane, normal emulsions, gels, ointments, creams or mixed phase and/or amphiphilic emulsion systems (oil/water-water/oil mixed phase) as well as liposomes and transfersomes can be named. Sodium algenate as a gel builder for production of a suitable foundation or cellulose derivatives, such as for example guar or xanthene gum, inorganic gel builders, such as for example aluminum hydroxides or bentonites (so-called thixotropic gel builder), polyacrylic acid derivatives, such as for example Carbopol®, polyvinylpyrolidone, microcrystalline cellulose or carboxymethylcellulose are suitable as adjuvents and/or carriers. Furthermore, amphiphilic low and high molecular weight compounds as well as phospholipids are suitable. The gels can be present either as hydrogels based on water or as hydrophobic organogels, for example based on mixtures of low and high molecular paraffin hydrocarbons and vaseline.

[0076] Anionic, cationic or neutral tensides can be employed as emulsifiers, for example alkalized soaps, methyl soaps, amine soaps, sulfanated compounds, cationic soaps, high fatty alcohols, partial fatty acid esters of sorbitan and polyoxyethylene sorbitan, for example lanette types, wool wax, lanolin, or other synthetic products for the production of oil/water and/or water/oil emulsions.

[0077] Hydrophilic organogels can be formulated, for example, on the basis of high molecular polyethylene glycols. These gel-like forms are washable. Vaseline, natural or synthetic waxes, fatty acids, fatty alcohols, fatty acid esters, for example as mono-, di-, or triglycerides, paraffin oil or vegetable oils, hardened castor oil or coconut oil, pig fat, synthetic fats, for example based on acrylic, caprinic, lauric and stearic acid, such as for example Softisan® or triglyceride mixtures such as Miglyol® are employed as lipids in the form of fat and/or oil and/or wax-like components for the production of ointments, creams or emulsions.

[0078] Osmotically effective acids and bases, such as for example hydrochloric acid, citric acid, sodium hydroxide solution, potassium hydroxide solution, monosodium carbonate, further buffer systems, such as for example citrate, phosphate, Tris-buffer or triethanolamine are used for adjusting the pH value.

[0079] Preservatives, for example such as methyl- or propyl benzoate (parabenes) or sorbic acid can be added for increasing stability.

[0080] Pastes, powders or solutions are to be mentioned as further topically applicable forms. Pastes often contain lipophilic and hydrophilic auxiliary agents with very high amounts of fatty matter as a consistency-giving base.

[0081] Powders or topically applicable powders can contain for example starch varieties such as wheat or rice starch, flame dispersed silicon dioxide or silica, which also serve as diluents, for increasing flowability as well as lubricity as well as for preventing agglomerates.

[0082] Nose drops or nose sprays serve as nasal application forms. In this connection, nebulizers or nose creams or ointments can come to use.

[0083] Furthermore, nose spray or dry powder formulations as well as controlled dosage aerosols are also suitable for systemic administeration of the active ingredients.

[0084] These pressure and/or controlled dosage aerosols and dry powder formulations can be inhaled and/or insufflated. Administration forms of this type also certainly have importance for direct, regional application in the lung or bronchi and larynx. Thereby, the dry powder compositions can be formulated for example as active ingredient-soft pellets, as an active ingredient-pellet mixture with suitable carriers, such as for example lactose and/or glucose. For inhalation or insufflation, common applicators are suitable which are suitable for the treatment of the nose, mouth and/or pharynx. The active ingredients can also be applied by means of an ultrasonic nebulizing device. As a propellant gas for aerosol spray formulations and/or controlled dosage aerosols, tetrafluoroethane or HFC 134a and /or heptafluoropropane or HFC 227 are suitable, wherein non-fluorinated hydrocarbons or other propellants which are gaseous at normal pressure and room temperature, such as for example propane, butane or dimethyl ether can be preferred. Instead of controlled dosage aerosols, propellant-free, manual pump systems can also be used.

[0085] The propellant gas aerosols can also suitably contain surface active adjuvents, such as for example isopropyl myristate, polyoxyethylene sorbitan fatty acid ester, sorbitan trioleate, lecithins or soya lecithin.

[0086] For regional application *in situ*, solutions for installation, for example for transurethral administration in bladder tumors or genital tumors, or for profusion in liver tumors or other organ carcinomas are suitable.

Literature references from Table 1

[0087]

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Claims

1. Biologically active substance which inhibits the cellular formation of niacinamide mononucleotide.

2. Biologically active substance according to claim 1 having an inhibitory activity on cellular NAD biosynthesis from the precursor niacinamide at concentrations of ≤ 10 μM of 50 %, preferably 80 %, most preferably 90 %.

3. Biologically active substance according to claim 1 or 2 which is an inhibitor of niacinamide phosphoribosyl transferase.

4. Biologically active substance according to claim 1 or 2 which inhibits the transport of niacinamide across the cell membrane.

5. Biologically active substance according to claim 1 or 2 which is selected from:

1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-3-pyridine-3-yl-urea;
4-benzhydryl-piperazine-1-carboxylic acid-[6-(3-pyridine-3-yl-methylureido)-hexyl]-amide;
1-(3,3-diphenylpropyl)-3- [6-(3-pyridine-3-yl-methylureido)-hexyl]-urea;
1-[5-(1-benzhydryl-piperidine-4-yl)-pentyl]-3-pyridine-3-yl-thiourea;
6-(4-benzhydryl-piperazine-1-yl)-hexanoic acid-(2-pyridine-3-yl-ethyl)-amide;
1-(6,6-diphenyl-5-hexenyl)-3-(pyridine-3-yl-methylene-amino)- thiourea;

N-(4-{1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-piperidine-4-yl}-butyl)-3-pyridine-3-yl-propanoic acid amide;
1-[4-(1-benzhydryl-piperidine-4-yl)-butyl]-3- (2-pyridine-3-yl-ethyl)-urea;
N-{2-[5-(4-benzhydryl-piperazine-1-yl-methyl)-1-methyl-1H-pyrrole-2-yl]-ethyl}-3-pyridine-3-yl-acrylamide;
1-{4-[1-(naphthalin-2-sulfonyl)-piperidine-4-yl)-butyl}-3-pyridine-3-yl-urea;
1-{4-[1-(10,11-dihydro-dibenzene[b,f]azepine-5-carbonyl)-piperidine-4-yl]-butyl}-3-pyridine-3-yl-urea; and
2-amino-3-[4-hydroxy-3-(2-{4-[4-(3-pyridine-3-yl-acryloyl-amino)-butyl]-piperidine-1-carbonyl}-phenylazo)-phenyl]-prpanoic acid trihydrate.

6. Pharmaceutical composition comprising a biologically active substance according to any of the claims 1 to 5, or a pharmaceutically acceptable salt thereof, optionally together with pharmaceutically acceptable formulation additive.

7. Pharmaceutical composition according to claim 6 for the treatment of cancer or immunosuppression in mammals.

8. Pharmaceutical composition according to claim 7, wherein the cancer is selected from breast, prostate, lung, colon, cervix, ovary, skin, CNS, bladder, pancreas and leukemia and lymphoma.

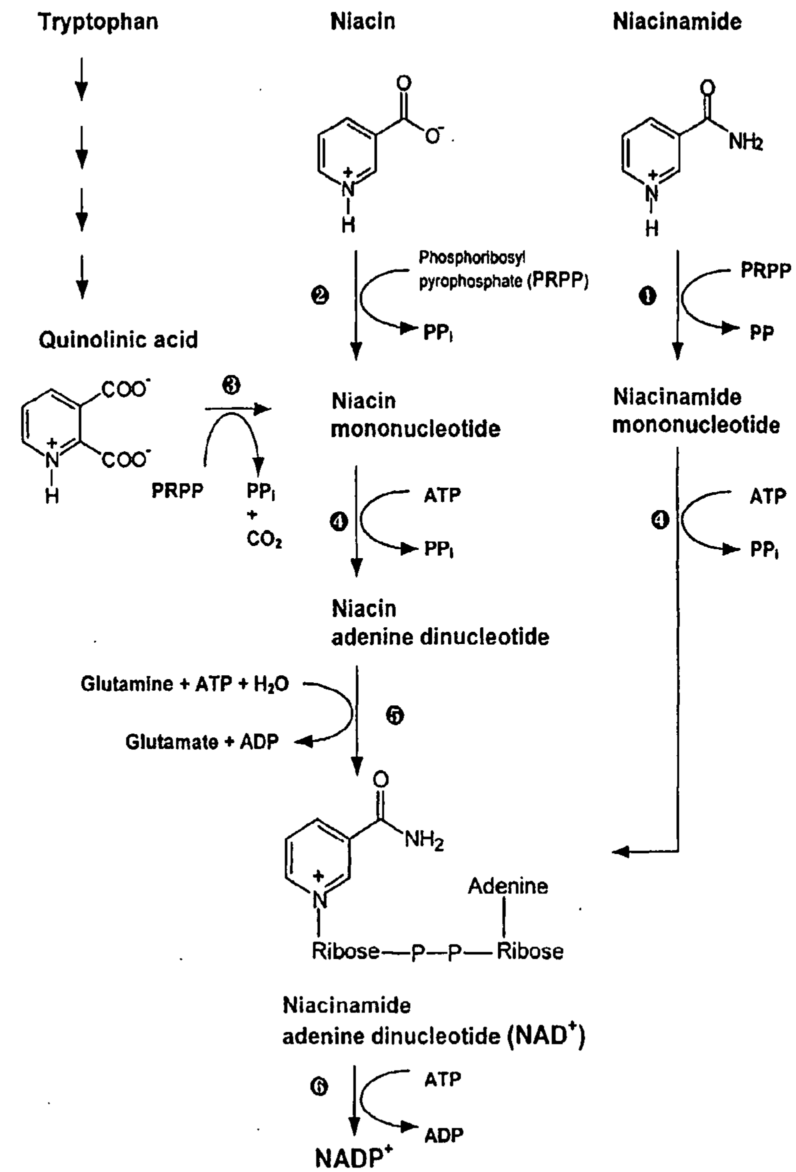
9. Pharmaceutical composition according to any of the claims 6 to 8 which is formulated for intraperitoneal, subcutaneous, oral, intravenous, rectal, buccal, intramuscular, intravaginal, topic or pulmonal administration.

10. Use of a biologically active substance according to any of the claims 1 to 5, or a pharmaceutically acceptable salt thereof, for the preparation of a pharmaceutical composition for the treatment of cancer in mammals.

11. Method for screening and detecting biologically active substances according claims 1 to 5 comprising:

    incubating cultured cells selected from HepG2 cells, U-87 MG cells, MCF-7 MI cells, Caki-1 cells, HL-60 cells, PC3 cells, U-373 MG cells, A549 cells and KG-la cells in the presence of [^{14}C]niacinamide and a substance to be tested for its activity to inhibit the cellular formation of niacinamide mononucleotide;
    effecting lysis of the cells;
    isolating and separating the [^{14}C]-labelled compounds and measuring the amount of formed labelled niacinamide mononucleotide, NAD and NADP.

12. Method for determining the dependency of a cell type on niacinamide as a precursor for NAD synthesis comprising: incubating cells to be assayed in the presence of a substance according to claims 1 to 5 in a medium containing only niacinamide as a NAD synthesis precursor; and perfoming a cytotoxicity assay after the incubation period.



1. niacinamide phosphoribosyl transferase
2. niacin phosphoribosyl transferase
3. quinolinic acid pyrophosphate phosphoribosyl transferase
4. NAD pyrophosphorylase
5. NAD synthetase
6. NAD kinase

Figure 1: Biochemical Pathways of NAD(P)$^+$ Biosynthesis

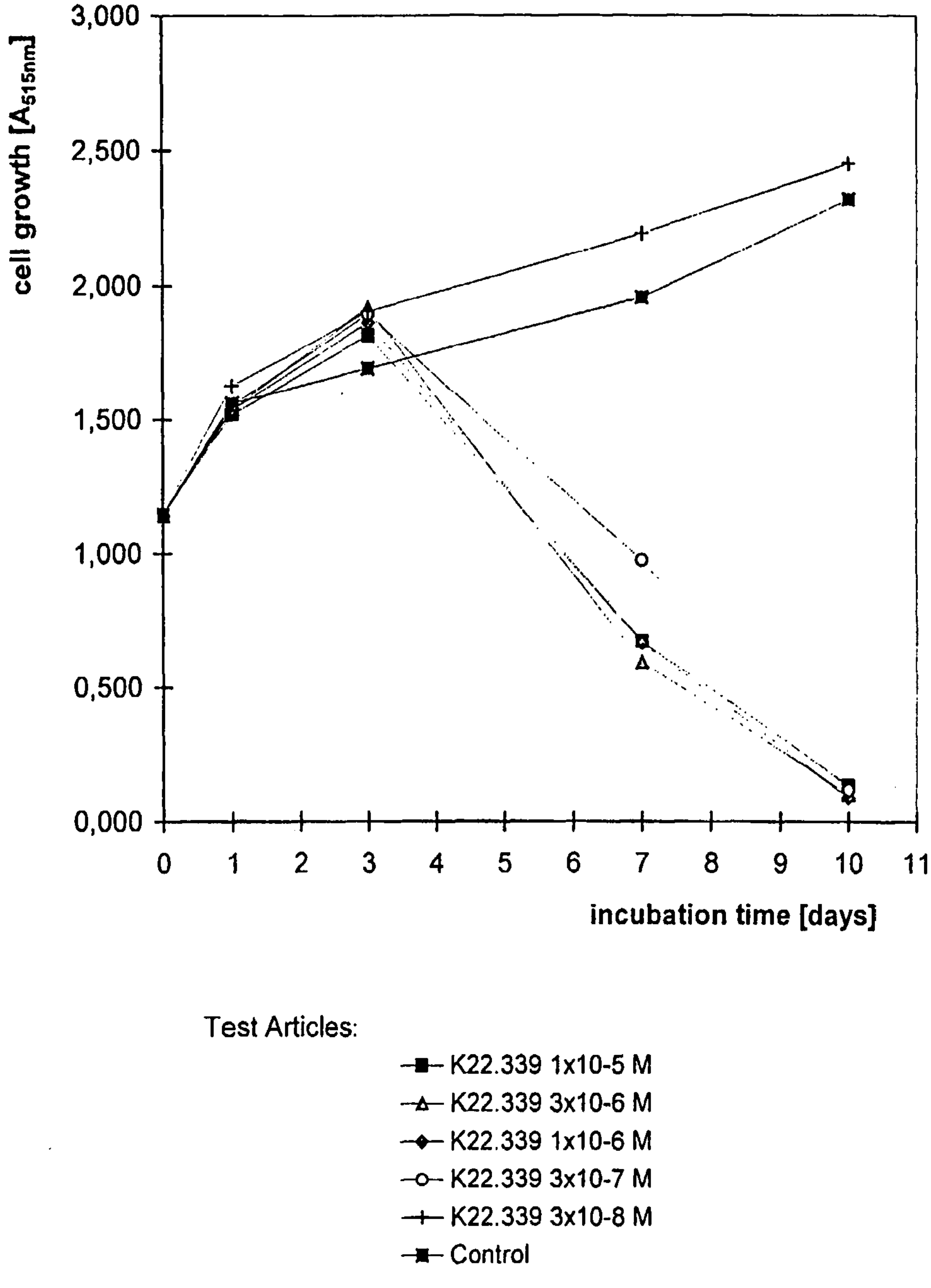
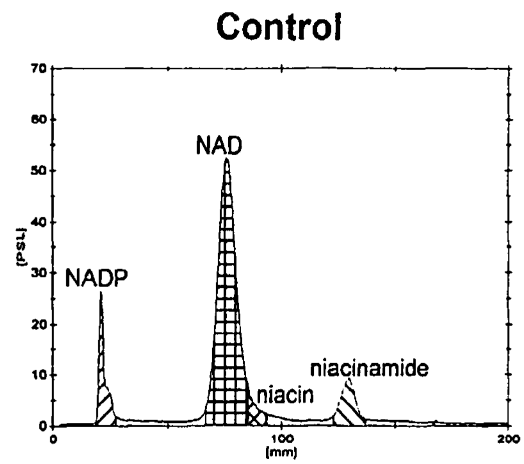


Figure 2: Induction of "delayed cell death" in HepG2 cells.



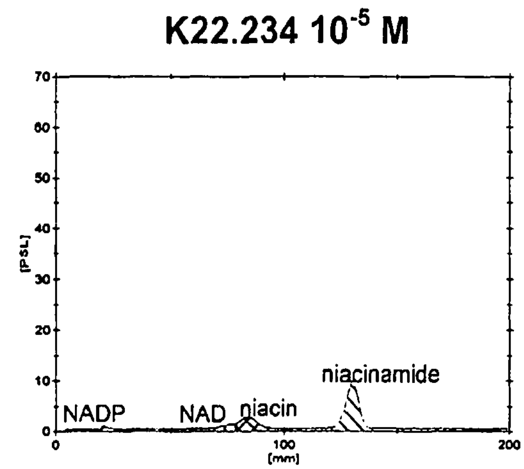


Figure 3: Inhibitory action of K22.234 on NAD(P) biosynthesis from [^{14}C]niacinamide in HepG2 cells. The radioactive metabolites of the cell extracts were separated on PEI cellulose using 0.05 M LiCl as solvent.

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PARTIAL EUROPEAN SEARCH REPORT

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

Application Number EP 99 10 3814

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	WO 97 48696 A (REITER FRIEDEMANN ;HASMANN MAX (DE); LOESER ROLAND (DE); BIEDERMAN) 24 December 1997 (1997-12-24) * Table 1; claims 18-40 *	5-10	C07D213/24 C07D213/38 C07D213/53 C07D213/89 C07D401/12 A61K31/44 A61K31/495 A61K31/55 C12Q1/68
Y	WO 97 48397 A (KLINGE CO CHEM PHARM FAB ;BIEDERMANN ELFI (DE); HASMANN MAX (DE);) 24 December 1997 (1997-12-24) * Tables 1 and 2; claims 1-18 *	5-10	

TECHNICAL FIELDS SEARCHED (Int.Cl.7)

C07D
A61K
C12Q

INCOMPLETE SEARCH

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely :

Claims searched incompletely :

Claims not searched :

Reason for the limitation of the search:

see sheet C

Place of search	Date of completion of the search	Examiner
MUNICH	6 September 1999	Uiber, P

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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INCOMPLETE SEARCH SHEET C

Application Number
EP 99 10 3814

Claim(s) searched completely:
5,11,12

Claim(s) searched incompletely:
1-4,6-10

Reason for the limitation of the search:

Present claims 1-4 and relate to a compound or composition or defined by reference to a desirable characteristic or property, namely:

- inhibition of the cellular formation of niacinamide mononucleotide

The claims cover all compounds or compositions or uses having this characteristic or property, whereas the application provides support within the meaning of Article 84 EPC and/or disclosure within the meaning of Article 83 EPC for only a very limited number of such compounds or compositions or uses. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 84 EPC). An attempt is made to define the product/compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds of claim 5, compositions containing the compounds of claim 5 or uses of the compounds of claim 5 according to claim 10 and the methods of claims 11 and 12.

ANNEX TO THE EUROPEAN SEARCH REPORT ON EUROPEAN PATENT APPLICATION NO. EP 99 10 3814

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report.
The members are as contained in the European Patent Office EDP file on
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06-09-1999

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